Amine Oxidases

MAO, and analysed the resulting enzyme activity obtained. The transfected cells expressed monoamine oxidase activity of the correct form showing that a single subunit is sufficient to generate activity and that the identified cDNAs code for enzymically active type A and type B monoamine oxidases.

The successful expression of the monoamine oxidase cDNAs in a mammalian cell enables the direct study of hypotheses of structure and function. The production of chimaeric forms of monoamine oxidase consisting of the N-terminal sequences of MAO-A and the C-terminal sequences of MAO-B, for example, could be illuminating. When these constructs are transfected into mammalian cells enzymic assays may reveal which domains of the MAOs determine the enzyme specificity. More specific site-directed mutation of individual amino acid residues will identify those amino acids forming the active site.


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The use of mechanism-based inactivators to probe the mechanism of monoamine oxidase

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Monoamine oxidase (MAO; EC 1.4.3.4) is one of the enzymes responsible for the catabolism of various biogenic amine neurotransmitters as well as for the metabolism of certain exogenous amines [1]. The enzyme exists in two different isozymic forms known as MAO-A and MAO-B [2], which differ in substrate specificity, distribution among tissues and in their structures [3–5]. Non-specific or MAO-A selective inhibitors have been shown to be clinically useful as antidepressants; the clinical usefulness of MAO-B-selective inhibitors in the treatment of depression remains to be demonstrated [6]. However, MAO-B-selective inhibitors are being used as adjuncts to the L-dopa treatment of Parkinson’s disease [7, 8]. Furthermore, the administration of MAO-B inhibitors alone early in the course of this disease can alleviate the need for other drugs, presumably by slowing the progression of the disease [7].

We have been interested in the use of mechanism-based inactivators [9] of MAO as a probe for the mechanism of this enzyme and in the design of new classes of inactivators. Mechanism-based inactivators have been shown to be quite useful in the study of enzyme mechanisms because they are really nothing more than substrates for an enzyme which happened to be converted into products that react with the enzyme and inactivate it. But since the mechanism by which these compounds are converted into the reactive products proceeds, at least initially, by the normal catalytic mechanism, any information that is obtained regarding the inactivation mechanism is directly related to the catalytic mechanism. That is the approach that we have taken to elucidate the mechanism for mitochondrial MAO.

MAO is a dimeric flavoenzyme in which one FAD is covalently attached to each subunit of the apoprotein [10]. It catalyses the oxidation of amines to the corresponding imines with concomitant

Abbreviation used: MAO, monoamine oxidase.

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reduction of the flavin. Little was known about the molecular mechanism of action of this enzyme when we became interested in it, but it is known from a variety of organic chemical studies of amine oxidation that chemical [11], electrochemical [12], and photochemical [13, 14] amine oxidations proceed by one-electron mechanisms. Consequently, we [15] and Krantz and his co-workers [16] proposed one-electron mechanisms for the enzyme reaction; a more comprehensive mechanistic scheme is shown in Fig. 1. One-electron transfer from the amino group to the flavin gives the amine radical cation which can lose a proton to give the carbon radical. This radical can be oxidized further either by second electron transfer or by radical combination with an active site radical (either the flavin radical just generated or an amino acid radical generated by hydrogen atom transfer from the amino acid to the flavin [17]) followed by β-elimination to the immonium product. Work is presented here that supports both of these possibilities.

On the basis of the work by Maeda & Ingold [18], a cyclopropyl substituent was selected as our first probe for a one-electron transfer mechanism. A variety of cyclopropylamines were synthesized and all were shown to be potent mechanism-based inactivators of MAO [15, 17, 19–27] which led to cyclopropyl ring opening and attachment to the enzyme. Since chemically-generated cyclobutylaminium radical cation analogues can undergo rearrangement reactions, a cyclobutylamine was incubated with MAO and the expected rearrangement was observed [28]. More direct evidence for a radical intermediate in MAO-catalysed amine oxidations was obtained by carrying out the reaction of MAO with poor substrates (cyclobutylamines) in the presence of a radical spin trap in an e.s.r. cell [29]. The expected triplet of doublets for the nitroxy radical was observed.

The above-mentioned evidence supports an initial one-electron transfer from the amine to the flavin. Recent studies with two other mechanism-based inactivators of MAO will be described that provide evidence for a second-electron transfer and both of the other pathways shown in Fig. 1. Several years ago we published our initial work on the design of (aminoalkyl)trimethylsilanes, a new class of mechanism-based inactivators of MAO [30]. We now have carried out detailed studies with one member of this class of compounds, namely, (aminomethyl)trimethylsilane (Me₃SiCH₂NH₂) (1). This compound has a structure identical with that of a substrate for MAO, namely, neopentylamine, except that a carbon atom is replaced by a silicon atom. Since neopentylamine is only a substrate, but (1) is an activator, the silicon atom must be responsible for the inactivation. Following inactivation, which occurs with concomitant reduction of the flavin, enzyme activity slowly returns, suggesting that a weak covalent bond is formed. Several inactivation mechanisms can be considered (Fig. 2). Following one-electron transfer to (2), an active-site nucleophile could react with the strongly electrophilic silicon [31–34] which leads to a trimethyl-
silylated enzyme (3), or (2) could undergo the 'normal' catalytic mechanism (at least, normal according to Fig. 1). This would produce (2a) which could either transfer the second electron and give an acylsilane immonium ion (4) that reacts with an active site nucleophile or (2a) could undergo radical combination to give inactivated enzyme (5).

Both potential inactivated enzyme adducts, (3) or (5) should be somewhat unstable to hydrolysis and, in fact, incubation of the inactivated enzyme in buffer results in a slow time-dependent reactivation of the enzyme. Addition of fluoride ion to the inactivated enzyme does not increase the rate of reaction. Since fluoride ion is known to rapidly desilylate trimethylsilyl heteroatom species, this is evidence in favour of (5) as the inactivated enzyme adduct. According to pathway A in Fig. 1 [1-2H2](1) should lead to inactivation with no incorporation of tritium into the enzyme and concomitant release of tritium as [3H]formaldehyde. Pathways B and C, however, should give MAO containing one equivalent of tritium and no tritiated formaldehyde. Both results were obtained: 1.2 equivalents of radioactivity was bound to the enzyme and [3H]formaldehyde was released. This suggests that both pathways are important; however, if formaldehyde can be obtained from pathway B then pathway A may be irrelevant. Since acylsilanes [the product of hydrolysis of (4)] are known to hydrolyse with rearrangement to aldehydes [34], formaldehyde could be generated by pathway B. The question of which pathway is relevant was answered with [1-2H2](1).

Inactivation of MAO by pathway A or C would produce dideuterated formaldehyde, but pathway B, following hydrolysis and rearrangement, would give monodeuterated formaldehyde. In addition, since substrates for MAO exhibit kinetic isotope effects on C-H bond cleavage, pathway B, but not pathway A or C, should exhibit a deuterium isotope effect on inactivation. Inactivation of MAO by (1) and by [1-2H2](1) exhibited a deuterium isotope effect ($k_{\text{react}}/k_{\text{init}}$) of 2.3 with no effect on the $K_i$. This is the same isotope effect as is observed for the oxidation of [1-2H2]tyramine [35] and [1-2H2]dopamine [36] and indicates that pathway B is responsible for inactivation of MAO by (1). Mass spectral analysis of the formaldehyde formed (as the 2,4-dinitrophenylhydrazone) showed that both monodeuterated (pathway B) and dideuterated (pathways A or C) formaldehyde were formed in the ratio of 3.5 to 1, respectively. Therefore, both pathway A or C and pathway B are operative, but pathway B is responsible for inactivation and pathway A or C results in turnover to product without inactivation. The observation of product from pathway A or C is strong support for a radical mechanism. Mariano and co-workers [37] recently carried out a photochemical model study with (diethylaminomethyl)trimethylsilane that gave similar results to these enzymatic studies with (1).

According to pathway B, inactivation should result in the incorporation of one equivalent of tritium from [1-3H](1) and one equivalent of 14C from [14C-methyl](1) (label in the trimethylsilyl group), and reactivation of inactivated enzyme should release both labels at the same rate with concomitant return of enzyme activity. Inactivation of MAO with these inactivators led to the incorporation of 1.2 and 3.3 equivalents of radioactivity, respectively. Presumably, the additional two equivalents of 14C that are incorporated are trimethylsilylated enzyme nucleophiles. Incubation of the labelled enzymes at pH 7.0 resulted in pseudo first-order release of both 3H and 14C with half-lives of 29 h and 26 h, respectively, and the return of enzyme activity with a half-life of 21 h (three separate experiments; the latter experiment done with a different batch of enzyme than the other two). These results further support adduct (5). By recording the absorption spectrum of the flavin after inactivation and after denaturation of inactivated enzyme it was apparent that attachment of the adduct was to an amino acid residue, not to the flavin. The relevance of this adduct to the mechanism of MAO-catalysed amine oxidation is discussed at the end.

A class of MAO inactivators for which no mechanistic information is known is the 5-(aminomethyl)-3-aryl-2-oxazolidinones [(6) in Fig. 3]. Structure–activity relationship studies of a variety of oxazolidinones uncovered several very potent inhibitors of MAO, some of which were MAO-A selective and some MAO-B selective [39]. The most potent of the MAO-B inhibitors, (6) [R = 3-chlorobenzyl] [40], also was found to be an irreversible inactivator [41]. On the basis of our previous studies on the mechanism of MAO and a chemical model study for the mechanism of (6) [42], the inactivation mechanisms shown in Fig. 3 were proposed. All of the proposed pathways proceed by initial one-electron transfer from the amine to the flavin to give the amine radical cation (7) followed by proton removal. Pathways A and C involve formation of radical (8) from which either heterolytic C–O bond cleavage (pathway A) or homolytic C–O bond cleavage (pathway C) can occur, followed by attachment of the resultant radicals to an active site radical to give (10) or (12), respectively.
Pathway B is an E2 type mechanism which could occur from the carbanionic character that is generated initially by removal of the α-proton from the amine radical cation (7). This pathway leads to the same radical intermediate formed by pathway A. All three pathways would result in the loss of CO₂ from the inactivator molecule and could lead to attachment of the remainder of the molecule to the active site [(10) or (12)].

Several analogues of (6) containing radioactive labels were synthesized to test the proposed mechanisms with MAO. R- and S-[³H-methoxy]-(6) (R = 3-chlorobenzyl) inactivated MAO with the incorporation of 1.0 and 1.2 equivalent of tritium, respectively, after denaturation. The most important test of the validity of the mechanisms shown in Fig. 3 is whether the carbonyl group of the oxazolidinone is released as CO₂ during inactivation. Consequently, R- and S-[carboxy-¹⁴C]- (6) (R = 3-chlorobenzyl) were synthesized and inactivation of MAO were carried out in a closed vessel containing a base trap to collect any ¹⁴CO₂ that was generated. The R- and S-isomers inactivated the enzyme with release of 4.5 and 3.0 equivalents, respectively, of ¹⁴CO₂. Loss of CO₂ should be a measure of the MAO-catalysed oxidation of (6) (R = 3-chlorobenzyl) by one of the mechanisms in Fig. 3. Intermediates (9) and (11) may be released then reduced or oxidized, thereby accounting for the greater than one equivalent of CO₂ produced during inactivation. An alternative oxidation pathway is the normal oxidation reaction, namely, its conversion to the corresponding imine followed by hydrolysis to the aldehyde, a known metabolite of (6) oxidation [43]. According to the proposed inactivation mechanisms, inactivated enzyme should not contain ¹⁴C from [carboxy-¹⁴C]-(6), since this is lost as ¹⁴CO₂. However, inactivation of MAO with R- and S-[carboxy-¹⁴C]- (6) (R = 3-chlorobenzyl) led to the incorporation of 1.5 and 1.0 equivalents, respectively, of ¹⁴C per enzyme molecule! This indicates that decarboxylation is not involved in the mechanism of inactivation of MAO by (6), but it must be important to the MAO-catalysed metabolism of this class of inactivators. By recording the u.v.-visible spectrum of the flavin after inactivation
Proposed mechanism of inactivation of MAO by 5-(aminomethyl)-3-aryl-2-oxazolidinones

The mechanism proposed in [44] was oxidation of the amine to the imine by an unspecified pathway followed by attack of an active site nucleophile on the imine. This modified version takes into account previous evidence about the mechanism of the enzyme and accounts for the decarboxylation pathway and the known aldehyde metabolite formation.

![Diagram of proposed mechanism](image)

and denaturation it was apparent that attachment was not to the flavin, but rather to an amino acid residue at the active site.

On the basis of these results a possible inactivation mechanism is shown in Fig. 4. This a modification of the inactivation mechanism that was proposed by Dostert et al. [44]. The stability of (13) would be derived from the electron-withdrawing oxygen that is at the Β-position, since electron-withdrawing groups stabilize α- and β-sp²-carbons in preference to sp² carbons, presumably because of destabilization of the electron-deficient carbonyl. If that is the case, then, the oxazolidinone nitrogen is not important in the structure for inactivation. Consequently, the two corresponding diastereomeric lactones (6) (R=Me, no N in the ring) were synthesized. The trans-diastereomeric lactone is a better inactivator of MAO than is the corresponding oxazolidinone; the cis-lactone is a very poor inactivator. This supports the hypothesis that the β electron withdrawing group is the essential feature in the oxazolidinones that leads to inactivation after attachment to the enzyme. Since epimerization at the nitrogen of the oxazolidinone is a low energy process, and the trans-lactone is a much better inactivator than the cis-lactone, it is likely that one configuration of the oxazolidinone also is more active than the other.

Our results with (aminomethyl)trimethylsilane and 5-(aminomethyl)-3-aryl-2-oxazolidinones lend support for both pathways A and B in Fig. 1 as mechanisms of MAO-catalysed amine oxidation. Both of these compounds are analogues of typical substrates for MAO where R=trimethylsilyl and 3-aryl-2-oxazolidinon-5-yl, respectively. Proton removal from the amine radical cation of these substrates would give a carbon radical that could partition between second electron transfer (pathway A) to give the aldehyde metabolites [formaldehyde from (1) and the aldehyde metabolite of (6)] or radical combination with an active site amino acid radical (presumably a cysteiny radical [27]) to give a covalent intermediate (pathway B). In the cases of (1) and (6) this covalent intermediate is stabilized by the α-trimethylsilyl- and β-N-substituted 2-oxazolidinon-5-yl groups. Therefore, these compounds are acting as if they were normal substrates for MAO, but they lead to inactivation because the built-in electron-withdrawing groups stabilize the normal covalent intermediate in the enzyme reaction.

One might wonder why Mother Nature would be so careless as to design a redox enzyme with a cysteine residue near to the oxidizing flavin cofactor. It may not be carelessness, however, but a clever design element that allows MAO to catalyse oxidations of a variety of substrates having different second electron oxidation potentials. After oxidation proceeds to the carbon radical intermediate (Fig. 1), depending upon the oxidation potential of that species, the enzyme can continue along two different pathways. For those substrates with low oxidation potentials, second electron transfer may occur (pathway A), but for those substrates with high oxi-
dation potentials, radical combination and \( \beta \)-elimination could be an alternative route (pathway B). An electron-withdrawing group at the \( \beta \)-position of a substrate increases the oxidation potential for second electron transfer and would detour the mechanism to pathway B, which results in covalent adduct formation. Since thiolate is a better leaving group than the flavin for \( \beta \)-elimination, the rate of elimination would be accelerated by covalent bond formation to a cysteine residue rather than to the flavin.

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